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FURTHER INVESTIGATION INTO THE PRECIPITATION OF THE TYPHOID BACILLUS BY MEANS OF DEFINITE HYDROGEN-ION CONCENTRATION *

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The phenomenon of acid agglutination has been the subject of previous investigations by different workers with widely differing results. By acid agglutination is meant the spontaneous clumping of bacteria and other small particles in suspension by means of a definite concentration of the hydrogen ion.

It has been the contention of certain German workers that as definite concentrations have been proved specific for the precipitation of certain organisms, they might therefore be used as a differential test for recognizing certain bacteria; for example, B. typhosus. Other investigators have denied the validity of such conclusions and discouraged the idea that it could ever be of any practical use.

It has been the purpose of our investigations to determine whether there exists a specific hydrogen-ion concentration for the precipitation of all strains of B. typhosus; and, if so, whether this phenomenon could be applied in precipitating B. typhosus from other germs in suspension, and thus in many cases positively proving the presence of this bacillus in suspected waters.

Before giving the methods employed and the results of our investigations, we shall review the methods and conclusions of previous workers.

In the year 1911, Michaelis[1] investigated the phenomenon, using acetic acid. A series of solutions with gradually increasing amounts of the hydrogen ion was prepared as follows:

Solutions	N/NaOH	N/Acetic	Distilled Water	(H)
1	5 c.c.	7.5 c.c.	87.5 c.c.	1×10^{-5}*
2	5 c.c.	10 c.c.	85 c.c.	2×10^{-5}
3	5 c.c.	15 c.c.	80 c.c.	4×10^{-5}
4	5 c.c.	25 c.c.	70 c.c.	8×10^{-5}
5	5 c.c.	45 c.c.	50 c.c.	16×10^{-5}
6	5 c.c.	85 c.c.	10 c.c.	32×10^{-5}

* 1×10^{-5} is the chemical formula for indicating the hydrogen-ion concentration. Written in full it is 0.00001.

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[1] Deutsch. med. Wchnschr., 1911, 37, p. 969.

When a suspension of a 24-hour culture of B. typhosus was added to each of the solutions of this series, it was found that the suspension was always precipitated between Solutions 2 and 5 and that the optimal precipitation took place in Solution 3. Therefore, the optimal precipitation took place at a definite hydrogen-ion concentration which, according to Michaelis,[1] was 4×10^{-5}. B. coli was not precipitated in any of the solutions. B. paratyphosus was precipitated in Solutions 5 and 6. B. enteritidis was found to have no definite optimal precipitation point, but might be precipitated at any concentration, depending upon the strain used.

Beniasch,[2] who worked with very many strains of all the well-known pathogenic bacteria, using in each case acetic, lactic, and levulinic acid, concluded that each germ has a specific hydrogen-ion concentration for its optimal precipitation, that the optimal precipitation for B. typhosus is 3.6×10^{-5} regardless of the acid used, and that this concentration could be used for identifying B. typhosus.

Schidorsky and Reim3 have likewise claimed that this test is of differential value and they claim to have used it in practical work. Jaffé[4] denies that the test is specific and doubts whether it can be used to advantage. Sears[5] used hydrochloric acid, as well as acetic and lactic acid. He also worked with and without the sodium salt of the acid, with some difference, as a result, in precipitation. He found some strains of B. typhosus that were not precipitated anywhere near the (H) of Michaelis and Beniasch.

In our work we made use of 6 strains of B. typhosus. We also used 4 strains of B. coli, 2 strains of B. paratyphosus, 1 strain of B. paracoli, 1 strain of B. enteritidis, and 1 strain of B. dysenteriae. The strains of B. typhosus had all been in culture for a considerable time, most of them being known to be several years old. Altho the laboratory history of each strain indicated that it was a genuine typhoid bacillus, yet each one was carefully tested before beginning the investigations. They all gave typical growths and reactions upon gelatin, potato, milk, litmus lactose agar, dextrose broth, Dunham's peptone solution, nitrate broth, and Endo's medium. They were also agglutinated in immune sera, tho they were not all equally agglutinable.

The sources of the various strains of B. typhosus used were as follows: Strain 1010, isolated several years ago from a patient in the University of Colorado hospital, Boulder, Colorado, has been kept in culture in the department of bacteriology of the University of Colorado. Strain 1012 was secured from the laboratory of the Chicago board of health 5 years ago. It had been isolated from feces. Strain 1013 was secured from Parke-Davis Company, Detroit, Michigan, 2 years ago. Its previous history is not known. Strain 1014 was secured from the Cutler laboratory, Berkeley, California, about 1 year ago, previous history being unknown. Strain 1016 was recently secured from Dr. Philip Hilkowitz' clinical laboratory, Denver, Colorado, its previous history being unknown. Strain 1017 was secured from the Colorado state agricultural college, Fort Collins, Colorado, its previous history being unknown. It is not likely that these strains of B. typhosus are closely related, and therefore they may be regarded as fairly representative of all strains of this organism. We were unable to determine whether the sources of the various strains used by previous workers were thus widely separated, or whether the strains had been isolated from patients of one epidemic, or from one community.

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[2] Ztschr. f. Immunitätsf., 1912, 12, p. 241.

[3] Deutsch. med. Wchnschr., 1912, 38, p. 1125.

[4] Arch. f. Hyg., 1912, 76, p. 1.

[5] Proc. Soc. Exper. Biol. and Med., 1913, 10, p. 120.

The work was begun by using tartaric acid. In all the previous work the sodium salt was used to reduce the hydrogen-ion concentration and also to hasten the precipitation. (No explanation has been given as to why the salt hastens precipitation.) So we used a salt of tartaric acid,—sodium-potassium-tartrate. The acid and tartrate were carefully prepared in N/1, N/10, and N/100 solutions and a system of dilutions worked out in test tubes giving the alleged optimal hydrogen-ion concentration, with higher and lower concentrations on either side. Several such series of test tubes were set up and tested with B. typhosus. Tho there were some positive results, in the main they were irregular and not at all reliable.

Acetic acid was then substituted for tartaric acid for the following reasons: First, the hydrogen-ion concentration constant of tartaric acid is much higher than that of the acetic acid, and therefore more difficult to control; second, within 24 hours after setting up the system a fungus growth appears in tartaric acid which interferes with normal slow precipitation. The fungus growth brings down all germs by acting as a filter. This fungus growth can be prevented only by aseptic care.

In working with acetic acid and sodium acetate a system was set up identical with that used by Beniasch.[2] The solutions were prepared to give varying hydrogen-ion concentrations in a series. Table 1 indicates the amounts added to give the (H) in each tube:

The concentration of the hydrogen ion was computed from the formula $(H) = K \times (\text{acid})$, in which "K" equals the dissociation constant. The constant of acetic acid at room temperature is 1.8×10^{-5} or 0.000018. By solving for each solution in the series the hydrogen-ion concentration is determined for each tube. These are given in the table.

TABLE 1

SERIES OF TUBES — VARYING (H)

Solutions	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9
N/10 c.c. sodium acetate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Acetic acid c.c. N/10 Tubes 1-5, N/1 Tubes 6-9	0.06	0.12	0.25	0.5	1	0.2	0.4	0.8	1.6
Distilled Water	1.54	1.48	1.36	1.1	0.6	1.4	1.2	0.8	0
24-hr. culture suspension	1	1	1	1	1	1	1	1	1
Total fluid	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1
K- 1.8×10^{-5}	.....	.....	...	.....	.....	.....	......	.....	.....
Hydrogen-ion concentration—(H) ...	2.3×10^{-6}	4.5×10^{-6}	9×10^{-6}	1.8×10^{-5}	3.6×10^{-5}	7.2×10^{-5}	1.45×10^{-4}	2.9×10^{-4}	5.8×10^{-4}
B. coli 1002..........	.....	.....	...	.....	.....	.....	......	.....	.....
B. coli 1003..........	.....	.....	...	.....	.....	.....	......	.....	.....
B. coli 1004..........	.....	.....	...	.....	.....	.....	......	.....	.....
B. coli 1005..........	.....	.....	...	.....	.....	.....	......	.....	.....
B. paracoli 1008.....	.....	.....	...	.....	.....	.....	......	.....	.....
B. paratyphosus 1021	.....	.....	...	.....	.....	.....	......	.....	+
B. paratyphosus 1023	.....	.....	...	.....	.....	.....	......	.....	.....
B. dysenteriae 1024..	.....	.....	...	.....	.....	.....	......	.....	.....
B. enteritidis 1026...	.....	.....	...	.....	.....	.....	......	.....	.....
B. typhosus 1010....	.....	.....	...	.....	.....	.....	......	.....	.....
B. typhosus 1012....	.....	.....	...	.....	.....	.....	......	++	++
B. typhosus 1013....	.....	.....	...	++++	++++	.....	......	.....	.....
B. typhosus 1014....	.....	.....	...	+	++++	+++	+	.....	.....
B. typhosus 1016....	.....	.....	...	.....	++	++	+++	.....	.....
B. typhosus 1017....	.....	.....	...	+++	+++	+	+++	.....	.....

The agar used was prepared from Fairchild's culture peptone 1.5% acid. Before inoculating the tube, the water of condensation was poured off to avoid the salt that was in the media. The tubes were inoculated and incubated at 37.5 C. for from 20 to 30 hours, tho as a rule for just 24 hours. A few were incubated for 48 hours.

When the solutions were prepared, the growth from each agar slant was washed off with distilled water and filtered through filter paper to make sure that there were no small particles of agar in the suspensions. About 15 c.c. of the suspension were made from each tube. The suspension was then added to each test tube as indicated in Table 1. Then the series of tubes was placed in the incubator for 1 hour at 37.5 C., after which they were removed and the precipitate noted.

Each of the four strains of B. coli was thus treated, but no precipitate occurred either immediately or after several days' standing at room temperature. This was repeated 4 times with the same result each time.

The six strains of B. typhosus were also prepared and set up in the same manner, but with quite different results. Strain 1010 failed to be precipitated by any of the concentrations of the hydrogen ion used. This was repeated 3 times, but failed to give a precipitate at any time, either immediately or after several days' standing. Strain 1012 gave a precipitate in Tubes 8 and 9 after several hours' standing, but never immediately after removing from incubator. Twice it gave no macroscopic precipitation, but microscopically it showed agglutination. This strain, therefore, was slowly and poorly agglutinated and only at relatively high concentrations. Strain 1013 gave a very definite and rapid precipitation in Tubes 4 and 5. It was marked to the naked eye immediately upon removal from incubator. At first, it was in suspension in macroscopic masses, but soon fell to the bottom of the tube. This strain gave the same result upon 3 subsequent trials. Strain 1014 was likewise rapidly and definitely precipitated, but at a slightly higher concentration. Three times it was precipitatetd in Tubes 5 and 6, and once in 4 and 5. Strain 1016 was precipitated slowly, but definitely, within 24 hours. Once it was precipitated in Tube 7 only, and twice in Tubes 5 and 6 as well as 7. Strain 1017 was always rapidly precipitated in 4 and 5. Later it was precipitated in higher concentrations, beginning in 7 before 6,—a very interesting phenomenon which held good each time tried.

B. dysenteriae was tried twice, but was not precipitated anywhere in the system either early or late. The two strains of B. paratyphosus were tried 3 times, but in only one case was there a precipitation and that was in Tube 9 of Strain 1021 after 24 hours' standing. B. paracoli was never precipitated.

With this test we found that one strain of typhoid bacillus acted just like all the strains of B. coli, one was very similar to one strain of B. paratyphosus, while the other four were precipitated at fairly constant hydrogen-ion concentrations. One strain was not precipitated within 2.3×10^{-6} and 5.8×10^{-4} (H). Another strain was precipitated only at 2.9×10^{-4} and 5.8×10^{-4} (H). The four remaining strains were precipitated between 1.8×10^{-5} and 1.45×10^{-4}. These four strains could all be precipitated with 3.6×10^{-4}, if enough time were given.

Each strain was fairly constant in concentration necessary for precipitation. The slight variations can be accounted for in that it is quite impossible to obtain each solution with exactly the same hydrogen-ion concentration every time set up.

It was also observed that those strains which were most rapidly precipitated correspond with the strains which have the laboratory history of being the most rapidly and strongly agglutinated by immune typhoid sera. Strain 1010 is almost useless for agglutination tests with typhoid bacilli. It is likewise unprecipitable in any of the concentrations of the hydrogen ion prepared in the system.

These results agree with those of Sears,[5] but do not agree entirely with the findings of Michaelis[1] and Beniasch.[2] The reason for this disagreement must indicate that these earlier workers employed strains which were closely related, while we, like Sears, worked with strains very distantly related.

The foregoing facts render the application of this phenomenon to the precipitation of B. typhosus from suspected water rather discouraging. However, a few tests were made. To 500 c.c. of distilled water we added the acid and acetate in such proportions as to give 3.6×10^{-5}. Then we added the entire growth from a 24-hour agar slant of B. typhosus 1014 and incubated the whole for 1 hour, as we had done with test-tube preparations. The typhoid bacilli were precipitated, but only after several days, while in the test tube they were precipitated immediately. This delay was probably due to the fact that the suspension was far more dilute than in test tube. In water contaminated with B. typhosus, the bacilli are probably still more diluted than in this test preparation. Besides, these "wild" strains are said to be less agglutinable than those kept for a long while in culture. Moreover, as has been shown by our previous experiments, there is a different concentration for different strains of B. typhosus. Therefore, further work along this line was not pursued.

In making microscopic study of the suspensions of the strains that were not precipitated rapidly and at the supposed optimal precipitation point, we often observed that the bacilli were clumping very like bacilli in an agglutination test with typhoid bacilli, tho they had not yet agglutinated in large enough masses to be precipitated out of the solution. This fact would indicate that pseudo-clumping in an agglutination test with typhoid bacilli might easily be due to traces of acid washed into the hanging drop from the apparatus used in its preparation.

CONCLUSIONS

There is no definite hydrogen-ion concentration which will precipitate all strains of B. typhosus.

Tho there is a fairly wide range in hydrogen-ion concentration for the precipitation of the various strains of B. typhosus, most strains can be precipitated in the presence of 3.6×10^{4} hydrogen-ion concentration, derived from the dissociation of acetic acid in the presence of sodium acetate.

Those strains that are easily and rapidly agglutinated by means of immune sera are likewise easily and rapidly precipitated by the hydrogen ion, probably of the concentration 3.6×10^{-4}.

The hydrogen-ion test may sometimes aid in the determination of B. typhosus. However, it can never be a certain test.

It cannot be used as a practical method of precipitating B. typhosus from contamiated waters.

It indicates a possible source of error in performing an agglutination test with typhoid bacilli.